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CHAPTER 7

Combating biothreat pathogens: ongoing efforts for countermeasure development and unique challenges

Allen J. Duplantier[a,b], Amy C. Shurtleff[a,d], Cheryl Miller[a,c], Chih-Yuan Chiang[a,b], Rekha G. Panchal[a], Melek Sunay[a,e]

[a]Countermeasures Division, US Army Medical Research Institute of Infectious Diseases, Frederick, MD, United States
[b]Cherokee Nation Assurance, Frederick, MD, United States
[c]National Research Council (NRC) Research Associateship Program at USAMRIID, Washington, DC, United States
[d]The Geneva Foundation, Tacoma, WA, United States
[e]Oak Ridge Institute for Science and Education (ORISE) Fellowship Program at USAMRIID, Oak Ridge, TN, United States

Abbreviations

AMPK AMP-activated protein kinase
BoNT botulinum neurotoxin
Cat L cathepsin L
CCHF Crimean–Congo hemorrhagic fever
CDC Centers for Disease Control and Prevention
EBOV Ebola virus
EEEV eastern equine encephalitis virus
EF edema factor
ELISA enzyme-linked immunosorbent assay
EPEC enteropathogenic *Escherichia coli*
ER endoplasmic reticulum
FDA Food and Drug Administration
FRET fluorescence resonance energy transfer
GFP green fluorescent protein
GP glycoproteins
HC heavy chain
HCI high-content imaging
HDT host-directed therapy
HTS high-throughput screening
IND Investigational New Drug
IRE1α Inositol-requiring enzyme 1
JUNV Junin virus
LASV *Lassa virus*
LC light chain
LF lethal factor
LPS lipopolysaccharide
LVS live vaccine strain
mAb monoclonal antibody
MIC minimum inhibitory concentration

Drug Discovery Targeting Drug-Resistant Bacteria. http://dx.doi.org/10.1016/B978-0-12-818480-6.00007-2
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MNGC multinucleated giant cell
NHP nonhuman primate
NIAID National Institute of Allergy and Infectious Diseases
NPC1 Niemann–Pick C1
ODN oligodeoxynucleotide
PA protective antigen
PK pharmacokinetic
RNAi RNA interference
rNAPc2 recombinant nematode anticoagulant protein c2
RNP ribonucleoprotein
T3SS type 3 secretion system
TLR Toll-like receptor
TPP target product profile
US United States
VARV variola virus
VEEV venezuelan equine encephalitis virus
VGCC voltage-gated calcium channel
VLP virus-like particle
WEEV Western equine encephalitis virus
Y2H yeast two-hybrid

1 Introduction

The concept of bioterrorism and the intentional release of biothreat agents for purposes of harm to human and agricultural interests stimulates discussion of some unanswerable questions. Questions ranging from protection of a nation's security to military defense tactics, all point to the gravity of the problem for which scientists are working together in many areas of study such as the development of novel medical countermeasures to combat lethal infections, the prevention of the spread of disease in the general populace, and design of field-worthy diagnostic tools. A biothreat organism is generally thought to be one causing severe or lethal disease or has potential to induce panic over the prospect of infection therewith; one with high pathogenicity and/or contagious infectivity; one with strong environmental stability or probable transmission as an aerosol; one with ease of large-scale production for far-reaching dissemination; and one that can be controlled for directing the release to only the intended target rather than accidental harm to the perpetrator [1]. Improved preparedness for intentional release of bacteria, viruses, and toxins will not only protect military positions and strategies but will also increase ability to combat disease in naturally occurring epidemics of diseases caused by some of these organisms.

2 Biothreat agents

United States (US) government agencies, together with international government and health protection entities, have worked to classify bacteria, viruses, and toxins into Select Agent categories (https://www.selectagents.gov/SelectAgentsandToxinsList.html), and

Table 7.1 Select agents and toxins of concern as potential biothreats.

Agent	Disease
Current Tier 1 agents[a]	
[A]*Bacillus anthracis* and *B. cereus* biovar *anthracis*	Anthrax
[A]*Yersinia pestis*	Plague
[A]*Francisella tularensis*	Tularemia
[B]*Burkholderia mallei*	Glanders
[B]*Burkholderia pseudomallei*	Melioidosis
[A]*Clostridium botulinum* toxin, and *Clostridium* spp. producing the toxin	Botulism
[A]Ebola virus	Ebola virus hemorrhagic fever
[A]Marburg virus	Marburg virus hemorrhagic fever
[A]*Variola major* and *Variola minor*	Smallpox
Other agents of concern[b]	
[B]*Brucella* spp.	Brucellosis
[B]*Coxiella burnetii*	Q fever
[B]*Rickettsia prowazekii*	Typhus fever
[C]SARS- and MERS-associated coronaviruses	Severe acute respiratory distress
[C]Nipah and Hendra viruses	Viral encephalitis and respiratory disease
[A]Rift Valley fever virus	Rift Valley fever
[B]Venezuelan equine encephalitis virus	Encephalitis and fever
[A]*Lassa virus*	Lassa fever
[A]South American hemorrhagic fever arenaviruses (Junin, Machupo, Guanarito, Sabia, and Chapare)	Hemorrhagic fever
[A]Crimean–Congo hemorrhagic fever virus	Hemorrhagic fever

[A, B, C] Denote additional categorization into Category A, B, and C pathogens, per NIAID [5].
[a] Tier 1 agents of human pathogenicity are presented [4]. Two more Tier 1 agents are rinderpest virus and foot-and-mouth disease virus, which are of agricultural concern (not covered in this chapter).
[b] Important non-Tier 1 agents for which countermeasures are described in this chapter [4,5]. See www.selectagents.gov for a comprehensive list of non-Tier 1 Select Agents and Toxins. *SARS*, Severe acute respiratory syndrome; *MERS*, Middle East respiratory syndrome-related coronavirus.

the priorities for the development of medical countermeasures against these organisms have been defined through international discussions [2–4]. Currently classified as Tier 1 select agents are those pathogens of grave concern, whereas other useful classification categories are in use by US government entities such as National Institute of Allergy and Infectious Diseases and the US Centers for Disease Control and Prevention (CDC) list, denoting the pathogens as Category A, B, and C agents [5]. Various biothreat pathogens addressed in this chapter are grouped by general category and disease associated therewith (Table 7.1). There are many organisms on the CDC list; consequently, not all of them are addressed in this chapter [4]. The authors of this chapter have endeavored to provide a comprehensive survey of the literature and described the development

of medical countermeasures against high-priority bacterial and viral biothreat agents where the most progress has been made, and/or the most novel ground has been broken.

2.1 Bacterial biothreat agents

Bacteria cause disease in humans by invading tissue, altering the host immune response, and/or producing toxins or virulence factors. Many of the bacteria described here are difficult to treat clinically. The potential bacterial threat agents that pose the greatest risk to national security are ones that can be easily disseminated and result in high morbidity and mortality rates. The former Soviet Union is known to have weaponized at least 30 viral and bacterial agents, including several vaccine or drug-resistant strains [6]. Each agent has unique properties that present both a distinct threat and challenge for detection, prevention, and control.

Bacillus anthracis and *Clostridium botulinum* are Gram-positive bacterial agents of grave biothreat concern. *B. anthracis* is a spore-forming bacterium that causes cutaneous, respiratory, or intestinal forms of anthrax disease, which is an acute, rapidly progressing infection in any form. The *B. anthracis* spores are highly stable both in the environment and in the exposed individuals and can be easily disseminated via the aerosol route, thus making it a dangerous bacterium [7]. The anthrax attacks in 2001 caused widespread panic, damage, disease, and death, which increased national awareness to the threat of bioterrorism. The bacterium produces a lethal toxin that disrupts the host innate responses during the early stages of infection and ultimately leads to septicemia and death of the host (Fig. 7.1A). Antibiotic treatment requires a lengthy dosing regimen and is effective only if it is initiated during the early stage of the infection. Two monoclonal antibody (mAB)–based anthrax antitoxin therapeutics [Abthrax (raxibacumab) and Anthim (obiltoxaximab)] have been approved by the US Food and Drug Administration (FDA) and included in the Strategic National Stockpile for treating inhalational anthrax [8]. BioThrax, the only licensed anthrax vaccine, is indicated for preexposure prophylaxis of disease in persons at high risk of exposure and postexposure prophylaxis of disease following suspected or confirmed *B. anthracis* exposure [8]. Botulinum neurotoxin (BoNT), produced by *C. botulinum*, is extremely potent, lethal, and easy to produce, transport, and misuse. The toxin itself is the select agent, but the *Clostridium* organism, as an isolate capable of producing the toxin, is also classified as a Tier 1 select agent. There are seven serotypically distinct BoNTs (serotypes A–G) and they act by blocking neurotransmitter release and thereby preventing transmission of nerve impulses, which can lead to botulism, hallmarks of which are paralysis and respiratory arrest [9] (Fig. 7.1B). Current treatment is limited to Botulism Immune Globulin Intravenous, human-derived antibotulism toxin antibodies for the treatment of infant botulism types A and B, and Botulism Antitoxin Heptavalent (A–G), a mixture of immune globulin fragments developed from equine plasma for the symptomatic treatment of adult and pediatric botulism. The US Army has developed a similar antitoxin based on equine neutralizing antibodies that is

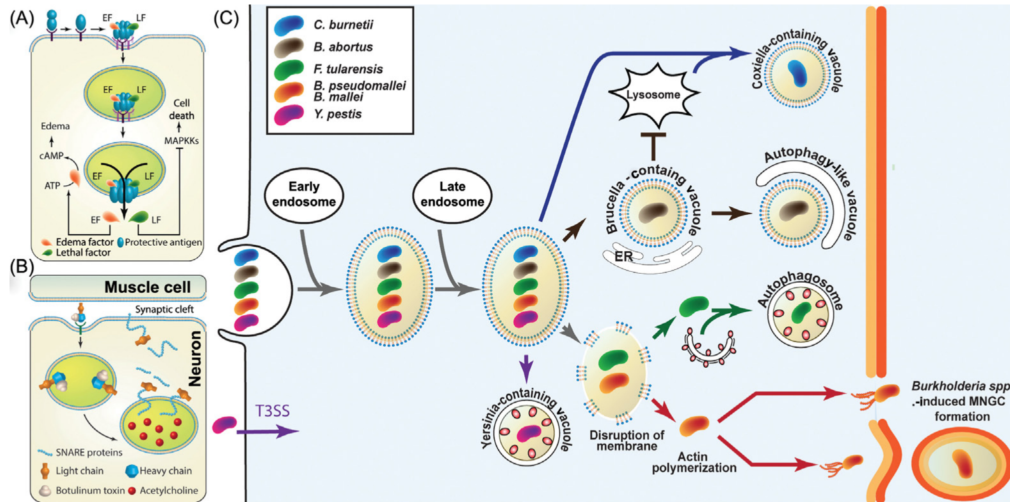

Figure 7.1 ***Mechanism of action of how bacterial pathogens invade, spread, and ultimately kill the mammalian host cell.*** (A) *Bacillus anthracis*, a spore forming Gram-positive bacterium secretes the three proteins—PA, LF, and EF. These proteins form a pore-forming heterocomplex that undergoes receptor-mediated endocytosis. The acidic environment in the endosomes causing a conformational change in the PA protein thereby resulting in the translocation of the LF and EF into the cytosol of the cell. LF is a Zn-dependent metalloprotease that is known to cleave several members of the mitogen-activated protein kinase kinase family, thereby preventing interaction with and phosphorylation of downstream MAPK and ultimately resulting in disruption of host signaling pathways. EF is a calmodulin-dependent adenylate cyclase that modulates host response by producing increased levels of cyclic adenosine monophosphate (cAMP) and causing severe edema in infected host [11]. (B) BoNTs are secreted by the sporulating and anaerobic Gram-positive bacteria of the genus *Clostridium*. BoNTs are produced as inactive single-chain polypeptides (150 kDa) that are cleaved by proteases to form the pharmacologically active toxin consisting of the LC and HC that are linked by disulfide bridges. The HC component binds to the receptors on the neurons and mediates toxin insertion. Inside the neurons the LC that is a Zn-dependent metalloprotease cleaves the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, thereby inhibiting the release of acetylcholine neurotransmitter into neuromuscular junctions and leading to neuroparalysis associated with botulism. (C) Intracellular bacterial pathogens share a number of mechanisms to enter, replicate, and disseminate; however, the repertoire of virulence factors that are unique to each pathogen dictate their intracellular niches. *C. burnetii* is unique in its ability to adapt the lysosome to create an ideal acidified vacuole for bacterial replication, called the Coxiella-containing vacuole. *Brucella abortus* is unique in its ability to acquire ER-derived membrane to create the Brucella-containing vacuole, where it can replicate. During late stages of infection *Brucella* spp. can convert vacuoles into autophagic vacuoles that facilitate bacterial egress and subsequent infections. *Francisella tularensis* can escape the vacuole and gain access to the cytosol of the cell where it can replicate to high numbers and late during infection in murine cells some cytosolic bacteria are found in autophagosomes and this population of surviving bacteria could be responsible for one mechanism of dissemination. *Burkholderia pseudomallei* and *Burkholderia mallei* also escape the phagosome and gain access to the cytosol where they replicate and spread from cell to cell using actin tails, resulting in the formation of MNGCs. *Yersinia pestis* is mainly an extracellular pathogen and secretes effectors using its T3SS; however, a few bacteria traffic intracellularly and reside within a Yersinia-containing vacuole that acquires autophagy markers, such as LC3. *BoNT*, botulinum neurotoxin; *EF*, edema factor; *HC*, heavy chain; *LC*, light chain; *LF*, lethal factor; *MNGC*, multinucleated giant cell; *PA*, protective antigen; *T3SS*, type 3 secretion system.

effective against a number of serotypes, but there is a limited supply and risk of horse serum sensitivity. An investigational vaccine also exists, but it offers limited protection and painful side effects [10].

Many of the bacterial agents of biothreat concern are intracellular Gram-negative organisms. Intracellular bacteria are particularly difficult to treat because the intracellular niche protects bacteria from the innate or adaptive immune surveillance. These bacteria can enter host cells through phagocytosis, and to prevent their destruction in the endocytic pathways, intracellular bacteria have adapted to survive in a host lysosome and replicate within the acidic endolysosomal compartment (e.g., *Coxiella burnetii*). Another intracellular bacteria *Brucella* spp. can traffic from a mature lysosome to endoplasmic reticulum–derived compartments, while bacteria such as *Burkholderia mallei*, *Burkholderia pseudomallei, Francisella tularensis*, *and Yersinia pestis* can prevent acidification and maturation of the phagosome and escape to the cytosol, where they can replicate and then disseminate to neighboring cells [12–14]. One characteristic feature of the *B. mallei* and *B. pseudomallei* intracellular life cycle is the fusion of infected mononuclear cells, forming multinucleated giant cells (MNGCs). Although the role of *B. pseudomallei*–induced MNGCs is unclear, it is believed that cell fusion facilitates localized dissemination of the bacteria [15,16] (Fig. 7.1C).

Brucella spp. are nonmotile bacteria that cause brucellosis, a world-wide chronic debilitating disease in both humans and animals. Although not typically fatal, *Brucella* spp. are stable and infectious as aerosols and can lead to sterility and abortions [17]. The nonmotile bacillus *B. mallei* is the causative agent of glanders that usually infects equids but is highly infectious to humans at low doses, producing septicemia, severe pulmonary infection, and chronic inflammation of the skin and eyes. *B. mallei* can be easily aerosolized, and even with antibiotic treatment there are high mortality rates [18]. The motile bacterium *B. pseudomallei*, the causative agent of melioidosis, is a close relative to *B. mallei* and can lead to severe illness in humans, such as pulmonary infection and septic shock. *B. pseudomallei* is an environmental saprophyte that is naturally resistant to many antibiotics [19]. Q-fever is caused by direct contact with the nonmotile bacterium *C. burnetii* that was previously weaponized because of its ease of aerosolization, its environmental stability, and its ability to infect animals or humans with a single bacterium [20]. Q-fever is not typically lethal but can be incapacitating, causing fever and difficulty breathing, and antibiotic therapy is not always effective, thus leading to persistent infections. *F. tularensis* is the causative agent of tularemia and is highly infectious, resulting in an acute, rapidly progressing local or systemic infection [21]. *Y. pestis*, the causative agent of plague, is a nonmotile bacterium that can be disseminated by aerosol, transmitted from person-to-person, and is characterized by a severe clinical disease course with potentially high case-fatality rates. There is a limited window for effective treatment against plague, since the resulting respiratory and circulatory collapse from septic shock is usually fatal [22].

2.2 Viral biothreat agents

There are a great number of viruses on the list of select agents and toxins, and some of these can only be handled in the laboratory at the highest biocontainment level (biosafety level 4). The causative agents of some of the most lethal hemorrhagic fever infections are filoviruses, paramyxoviruses, and arenaviruses. Filoviruses such as Ebola virus (EBOV) and Marburg virus infect humans and nonhuman primates (NHPs) and have caused large outbreaks in recent years. These viruses are likely transmitted in nature by fruit bats and are spread from person to person via contact with body fluids or fomites [23]. Marburg virus was reportedly weaponized through activities carried out by the former Soviet Union [24]. Because of the large scale of recent EBOV outbreaks, this virus may have become available to nefarious people through access to corpses and contaminated clinical waste. Two more viruses transmitted in nature at least in part by fruit bats are the Nipah and Hendra paramyxoviruses that belong to the Henipaviridae family and cause severe neurological and/or respiratory diseases in humans [25]. These viruses can infect many domestic and agricultural animal species and are frequently transmitted between humans via droplets or fomites, leading to concerns of new human outbreaks in areas of Malaysia, Bangladesh, India, and Australia where case-fatality rates range from 40% to 100% [26]. Arenaviruses, specifically *Lassa virus* (LASV) and Junin virus (JUNV), Machupo, and other South American viruses are transmitted not by bats but by peridomestic rodent species [27]. None of the filoviruses or henipaviruses has any FDA-approved therapeutics or vaccines available for prevention or treatment of human disease, and while ribavirin is sometimes used to treat Lassa fever, it is not a terribly effective drug against this viral infection [28].

Variola virus (VARV) is the causative agent of smallpox, a human viral disease for which tecovirimat was recently approved as a therapeutic by the FDA, a successful therapeutic development story [29]. This pathogen has been eradicated since 1979 through successful vaccine campaigns [30], but because the vaccine is no longer administered in most countries, populations may be susceptible in the event that VARV or an intentionally modified or related poxvirus with similar virulence factors and similar human lethality is resurrected [31].

Arthropod-transmitted alphaviruses and bunyaviruses are also biothreat concerns. For the alphaviruses specifically, the Venezuelan encephalitis viruses (VEEV), Eastern encephalitis viruses, and Western equine encephalitis viruses belonging to the family Togaviridae are found in the Americas and cause equine disease [32,33]. These new world alphaviruses cause encephalitis-like symptoms, are stable in the environment, grow to high titers easily in cell culture, are highly infectious by aerosol, and affect humans with incapacitating neurological disease, sometimes with high morbidity rates [33]. Bunyaviruses such as Rift Valley fever virus and the related tick-transmitted nairovirus causing Crimean–Congo hemorrhagic fever also cause human diseases with high morbidity and mortality rates. Some investigational new drug vaccines exist for these agents.

These viruses replicate quickly in humans and cause rapid disease; therefore the timing for therapeutic intervention is short, making treatment postinfection very challenging.

3 Screening strategies to identify therapeutics against biothreat agents

The lack of approved therapeutics available to combat biothreats may be in part attributed to the unique challenges for the discovery and development process of evaluating drugs that target select agents. Foremost is the implementation of high-throughput screening (HTS) efforts for the discovery of new compounds against authentic or wild-type biothreat bacterial and viral pathogens [34]. Specifically, the requirement of work to be performed in high-level biocontainment laboratories (BSL3 or BSL4) is a major limiting factor since laboratories with these capabilities are not widely available. In addition, highly trained personnel that can handle infectious agents use robotic instruments and adhere to operational, engineering, and government regulations are a critical requirement for working with biothreat agents [34]. In the United States, strict guidelines have been instated for generating government-approved methods and processes for inactivation of pathogens before plates/samples can be brought out of biocontainment suites for further experimentation, and to track the inactivated material [35]. Other challenges that need to be considered include the prevention of pathogen aerosolization while handling screening plates in biocontainment laboratories and ensuring that inactivation chemicals and methods are compatible with downstream procedures.

3.1 High-throughput screening approaches to identify therapeutics against bacterial agents

New therapeutics effective against both natural and engineered resistant forms of bacterium are vital to the biodefense armory. Screening for novel antimicrobials is traditionally done by scoring for growth inhibition in vitro, using the standard Clinical & Laboratory Standards Institute guidelines. This generally involves performing a dose–response assay in a multiwell plate format and monitoring growth in the absence or presence of the test compounds. The compound concentration that shows no visible growth is considered the minimum inhibitory concentration (MIC). Over the years, this approach has led to the discovery of only a limited number of novel antimicrobial compounds and resistance has already been generated against most of the antibiotics used in the clinic. One disadvantage to this approach is the inability to identify potent immunomodulatory compounds against intracellular pathogens that require the host for replication.

New approaches to understanding bacterial pathogenesis have enabled researchers to elucidate mechanisms that could be targeted to control and clear infection in lieu of simply targeting in vitro bacterial viability. Targeting the host under in vivo-like conditions (e.g., in cell culture or animal models) will be a key feature of study design to combatting

intracellular pathogens that require the host for invasion and replication and will likely identify new host-directed therapeutics. The development of host-directed therapeutic (HDT) strategy relies on an understanding of the interactions between pathogens and their hosts and appropriate tools and HTS assays to screen and identify therapeutics. Technological progress in assay miniaturization has emerged from a combination of advanced robotic systems, high-throughput microscopy, automated image analysis, and data analysis using powerful bioinformatics tools, and this has led to the development of high-content imaging (HCI), allowing for large-scale quantification of multiple cellular phenotypes at the system level. Such phenotypic screening platforms rely on physiologically relevant host cell types that are permissive to pathogen infection and have the potential to identify compounds that modulate relevant biological processes in an unbiased, target, and mechanism-agnostic fashion. This cell-based approach has the added advantage that compounds that have greater mammalian cell membrane permeability, reduced cellular toxicity, and target the host proteins will be readily identified in the context of their desirable function in cells. Pharmacologically active compounds can be selected that inhibit the uptake or intracellular replication of the bacterium or disrupt the host–pathogen interactions. The general workflow for high-throughput, image-based phenotypic screening approach to identify HDTs is outlined in Fig. 7.2 [36,37]. Using bacterial antigen-specific antibody to detect bacteria, this method can quantitate the number of intracellular or cell-associated bacteria and the effect of the compounds in reducing the bacterial number (% inhibition of bacterial infection), and cellular toxicity (based on loss in cell number). Alternatively, one can use HCI to quantitate the morphological changes of MNGCs based on nuclei number and MNGC size/area and use this phenotype to screen and identify compounds that prevent bacterial spread [38].

To overcome the problem of multidrug resistant bacteria, there is a growing focus on identifying small molecules that target drug resistant mechanisms or virulence factors, or agents that prevent/disrupt biofilm formation. Virulence factors, such as secretion systems in gram negative bacterial pathogens, are promising therapeutic targets. Specifically, the Type 3 Secretion System (T3SS) present in *Y. pestis* is responsible for injecting effectors that target the cytoskeleton and proinflammatory signaling pathways. A number of techniques have been used to screen and identify potential T3SS inhibitors that can be adapted for biothreat pathogens. These include an enzyme-linked immunosorbent assay (ELISA)-based detection of proteins secreted from enteropathogenic *Escherichia coli* (EPEC), inhibition of sheep erythrocyte lysis by EPEC, inhibition of induction of a *yopE* luciferase fusion in *Yersinia pseudotuberculosis*, and a *Pseudomonas aeruginosa* cell-based bioluminescent reporter screen [40–43]. Using a high-throughput luminescence screening assay, three compounds were identified that inhibit *Y. pestis* T3SS-mediated cytotoxicity that relieves the growth inhibition associated with in vitro activation of T3SS [44]. Another promising approach to disarm the bacteria is to prevent/disrupt biofilms, a barrier produced by bacteria to protect itself from the aggressive host environment.

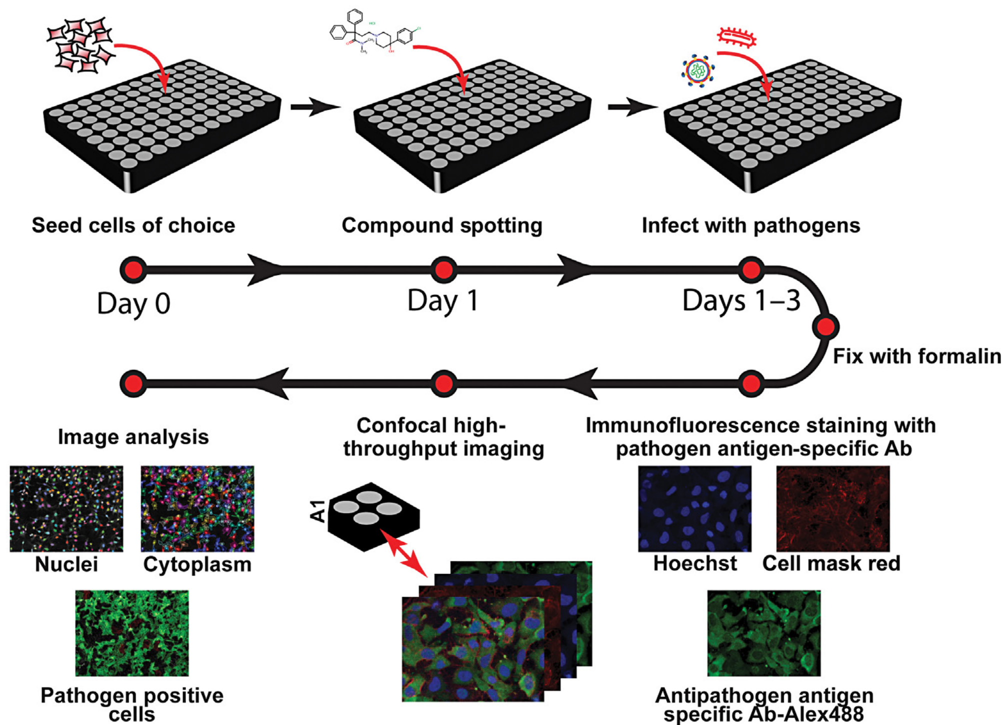

Figure 7.2 ***Phenotypic screening using high-throughput HCI.*** Cells susceptible to the pathogen of interest are seeded in HCI plates. Next day, cells are pretreated with appropriate concentration of the compounds and then infected with the pathogen of interest for optimal time wherein 70%–80% infection results. The infected plates are then submerged in 10% formalin for 24 h to inactivate the pathogen and to fix the cells. Immunofluorescence staining is then performed, using a primary antibody specific to a pathogen antigen, and an appropriate fluorescence-labeled secondary antibody. Dyes such as cell mask red and Hoechst are added to detect the cell cytoplasm and nuclei, respectively. Automated image acquisition and analysis is performed and data are analyzed using Columbus software to quantitate the percentage of inhibition of pathogen infection and loss in cell number that represents cellular toxicity, in the presence of the compound [39]. *HCI*, high-content imaging.

Small molecule therapeutics that specifically disrupt or prevent the biofilm formation could be used in combination with antibiotics. The common method to quantitate biofilms is a colorimetric-based assay that utilizes a crystal violet dye to stain the biofilms and subsequent extraction of the dye using organic solvents or detergents [45] followed by absorbance measurement. To improve sensitivity, robustness, and throughput, a fluorescent-dye-based assay was developed, wherein the biofilms are stained with FM1-43 fluorescent dye and fluorescence signal is measured following organic extraction of the dye [46]. Screening of a small molecule library in this assay identified rifabutin and ethavarine, as potential inhibitors of *B. pseudomallei* (Bp82) and *Acinetobacter baumannii* biofilm production, respectively, without directly affecting the bacterial growth.

There is a possibility that therapeutics targeting the virulence factors or other drug resistance mechanisms may not be effective by themselves and will need to be evaluated in combination with antibiotics to treat multiple drug resistance (MDR) infections. Thus screening experiments designed to find combination therapies are warranted. To determine the synergy of two drugs (antibiotic and nonantibiotic), conventional checkerboard assays are set up wherein the two drugs are tested in combination at varying concentrations and the MIC of each drug either alone or in combination is then used to calculate the fractional inhibitory concentration [47]. Similarly, in the case of the biofilm assay, testing a biofilm disruptor and an antibiotic together at varying concentrations will help one to assess the effectiveness of combination therapies.

3.2 Screening platforms for biothreat viral agents

Unlike most bacteria, viruses require the mammalian host for replication. The virus life cycle can be divided into distinct stages that include the entry, uncoating, replication, genome packaging, assembly, maturation, and budding. Various cell-based and in vitro biochemical assays have been developed to study virus life cycles as well as to screen and identify antivirals [48]. The conventional plaque-forming assay used to evaluate antivirals is time-consuming, not amenable for HTS, and not very robust. Alternatively, in the absence of more sophisticated instruments or technologies, a virus-induced cytopathic effect can be used as an endpoint to test antivirals. With advances in imaging instruments and informatics, a cell-based HCI platform (Fig. 7.2) that uses viral antigen-specific antibodies to detect and quantitate the viral infection is now a general approach to identify compounds that inhibit viral infection [39,49,50]. However, this approach will not provide information on which steps in the viral life cycle the inhibitors are disrupting. To help one to deconvolute the mechanism of action of identified hits (Fig. 7.3), cells pretreated with an inhibitor prior to virus exposure can potentially identify compounds that inhibit viral entry, while treatment of cells after exposure (i.e., after the entry step) would identify compounds that inhibit intracellular replication and/or viral spread.

Assays utilizing recombinant noninfectious viruses have been generated to screen and identify inhibitors that target different stages of the viral life cycle [48] (Fig. 7.3). Pseudotyped virion assays are well suited as safe alternatives for HTS, since BSL3 and BSL4 wild-type pathogens are not required to complete the screens. These assays are based on viral vectors that harbor glycoproteins (GPs) of different enveloped viruses and a reporter gene such as green fluorescent protein (GFP) or luciferase flanked by packaging signals, are used to generate chimeric replication-deficient viruses, and then used to screen and identify entry inhibitors. This approach has successfully identified entry inhibitors for Lassa, Ebola, and Nipah viruses [51–54]. Cell fusion assays, including cell–cell or cell–virus fusions, have been developed to screen and identify HIV-1 fusion inhibitors, but to date no such assays have been developed for biothreat viruses [55].

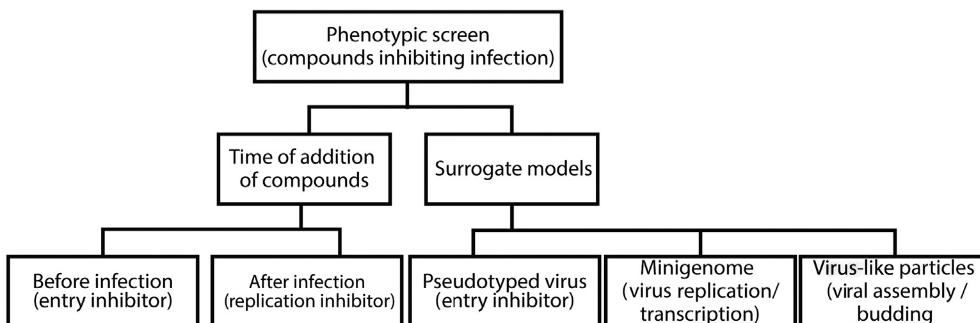

Figure 7.3 ***Phenotypic screening process. Methods to deconvolute the steps in the viral life cycle the hit compounds may be acting upon.*** Time of addition of the compounds will determine if compounds are affecting the entry or intracellular replication/spread of the virus. If cells are pretreated with the compound and then infected, then the hit compounds are possibly entry inhibitors. However, if the cells are infected and then treated with the compound, then the compounds may be affecting viral replication and/or budding. To further validate the hits during the various stage of the virus life cycle, surrogate models have been developed such as the use of pseudotype viruses to identify entry inhibitors, minigenome systems to identify hits that modulate virus replications/transcription or the VLPs to identify compounds that disrupt viral assembly/budding. *VLP*, virus-like particle.

Reverse genetic systems or minigenome assays have proven to be valuable models to study RNA virus replication and transcription. This model system is used to screen and identify antivirals [56,57]. Replication competent minigenome systems wherein some of the viral open reading frame is replaced with a reporter gene (GFP or luciferase) and the cDNA copy cloned into a plasmid is cotransfected into mammalian cells with individual plasmids each containing a viral ribonucleoprotein (RNP). The target genes in the expression vectors are under the control of either a mammalian RNA polymerase I or II or T7 RNA polymerase (which will require transfection of a plasmid containing the T7 RNA polymerase) promoters. Following transcription, the resulting viral RNA is complexed with the RNP components and there is subsequent replication of the virus genome and expression of the reporter protein. Minigenome systems have been developed for several biothreat pathogens, including filoviruses, arenaviruses, and bunyaviruses, and have been used to screen and identify small molecule inhibitors of filovirus and arenavirus replication [56–60]. To study the viral assembly and budding, another surrogate model that can be used is based on virus-like particles (VLPs) that are mimics of viral protein assemblies made by reconstituting the viral recombinant structural proteins. VLPs are noninfectious as they do not contain any viral genome, but are intrinsically immunogenic, and hence are being extensively investigated as potential vaccine candidates [61]. In the case of the EBOV VLP-based assay, cotransfection of plasmids encoding the viral GP and the matrix protein (VP40) results in spontaneous formation of filamentous VLPs that are released into the medium and can be quantitated by ELISA [62]; thus this model can be useful in drug discovery research [63].

To identify inhibitors of viral genome replication, in vitro biochemical assays targeting viral enzymes such as polymerases, methyltransferases, helicases, as well as viral and host proteases such as cathepsins or kinases have been developed. A number of antivirals that have been approved by the FDA target either the DNA or RNA polymerases. Incorporation of radioactive nucleotide either to a DNA oligonucleotide by DNA polymerase [64] or to a homopolymeric RNA as a template by RNA polymerase are common methods to determine polymerase activity [65]. A recent study reported the use of fluorescent dye to detect the double-stranded RNA and the feasibility of developing this assay to screen and identify inhibitors of Zika virus polymerase activity [66].

The host lysosomal protease cathepsin L (Cat L) is necessary for the processing and cleavage of the GP of enveloped viruses, so that the virus can fuse with the host cell membrane and gain entry into the host. Thus Cat L has been regarded as an ideal target for drug discovery. A fluorescence resonance energy transfer (FRET)-based Cat L enzymatic assay was developed, wherein peptides derived from GPs of viruses such as Ebola, Nipah, Hendra, and severe acute respiratory syndrome and Middle East respiratory syndrome coronavirus and containing Cat L cleavage site were chemically conjugated with a quencher 5-carboxytetramethylrhodamine at the N-terminus and 5-carboxyfluorescein fluorophore at the C-terminus [67]. The intact peptides exhibited minimal to no fluorescence, but following cleavage of the peptide by Cat L, there was an increase in fluorescence intensity. Screening of a chemical library in this assay identified small molecules that selectively inhibited Cat L-mediated cleavage of multiple viral peptides over host proneuropeptide Y [67]. Viral proteases are also good drug targets as they play a vital role in viral replication. For example, the NS2B-NS3 protease is highly conserved among the flaviviruses and a FRET-based enzymatic assay using a synthetic peptide substrate [68] was developed to identify West Nile virus protease inhibitors [69].

3.3 Identification of host factors required for pathogen replication through knowledge-based or multiomics screening

Functional genomic screening using gene-trapping, CRISPR's gene editing, or RNA interference (RNAi) technologies has been applied to identify host factors that are required for replication or involved in pathogenesis of several biothreat viral and bacterial agents and are summarized in Table 7.2. The activities of several identified host factors can be perturbed by small molecules and thus serve as potential therapeutic platforms. For example, it was demonstrated that the novel host factor inositol-requiring enzyme 1α is required for *Brucella* infection in mammalian cells [70]. Reducing the levels of either the retromer cargo-adapter complex or retromer-associated sorting nexins abrogated *C. burnetii* replication [71]. Multiple host kinases such as cAMP-dependent protein kinase, protein kinase B, and protein kinase C all play a role during *C. burnetii* infections [72,73]. Zhou et al. [74] identified TNFRSF9 and SERPINI1 that may

Table 7.2 Host factors identified using functional genomics screening technologies.

Pathogen	No. of genes being targeted in the library	Gene family target	Major pathways identified	Host factors identified	Reference
Bacterial					
Brucella spp.	240	ER-associated proteins	Inositol metabolism, eukaryotic unfolded protein response	IRE1	[70]
Coxiella burnetii	21,121	Whole genome	Retromer complex	VPS26, VPS29, VPS35	[71]
Francisella tularensis	~47,400 transcripts	Whole genome	Multiple pathways	TNFRSF9, SERPINI1	[74]
	~21,300	Drosophila Whole genome	Lysosomal fusion and phagosomal escape	PI4KCA, USP22, CDC27	[13]
Yersinia pestis	17,370	Whole genome	Endosome recycling	RAB4A	[14]
Viral					
Arenaviridae JUNV	9000	Druggable genome	Viral entry, ubiquitin ligase	VGCC, ARFRP1, CLDN2, CSDC2, KSR2, LTBP2, SSU72, TRIM2	[75]
Filoviridae EBOV	~800,000 insertions in introns	Whole genome	Fusion of endosomes and lysosomes/ biogenesis of endosomes, lysosomes, luminal cargo to the endocytic pathway, cholesterol transporter	NPC1, HOPS complex, CTSB, PIKEYVE, GNPTAB	[76]
	19,050	Whole genome	Sugar transporter involves in lysosome function, Zinc transporter protein, HOPS complex	GNPTAB, CTSB, CTSL, NPC1, SPNS1, SLC30A1, HOPS complex	[77]
	N/A	N/A	HOPS complex, endosome maturation, lysosomal protein	NPC1, CTSB, BRI3, FIG4, PIKFYVE, VPS16/39/41, RAB39B	[78]
	21,566	Whole genome	De novo pyrimidine synthesis pathway	CAD, NXF1, DDX39B	[79]
	720	Kinome	Proteins in PI3K and calcium/ calmodulin kinase related network	PI3K, CAMK2	[80]

Filoviridae MARV	21,585	Whole genome	biosynthesis of heparan sulfate, MARV entry, HOPS complex	CTSL, EXT1, NPC1, HPS complex	[81]
Paramyxoviridae Henipavirus	18,120	Whole genome	Ribosomal biogenesis, nuclear export/import, transcriptional regulation, rDNA transcription, pre-rRNA cleavage, subunit assembly, chemical modification of pre-rRNA	RPL, RPS, ESF1, XOP1, KPNA3, BTF3, SP7, POLR3E, DDX10, IMP4, GTPBP4, RPL13A, FBL	[82]
Poxviridae *Vaccinia virus*	6719	Druggable genome	Translation of mRNA, ceullar transcriptional processes, DNA repair pathways, AMPK complex, GTP binding proteins	153 pro- and 149 antiviral host factors	[83]
	~440	Drosophila kinome	AMPK pathway	PRKAA2, PRKAG2, PRKAB1, PIKFYVE, PIK3C2A, STAM, PTPN23, MYLK	[84]
	21,566	Whole genome	Translational, ubiquitin-proteasome, and ER-to-Golgi transport function, RNA polymerase II	NUP62	[85]
Togaviridae VEEV	140	Genes involved in trafficking	Actin rearrangements, trafficking in trans-Golgi network	Rac1, PIP5K1-a, Arp2/3 complex	[86]

promote activated macrophages in controlling *F. tularensis* replication. Akimana et al. [13] showed that *F. tularensis* utilizes host ubiquitin turnover in distinct mechanisms during the phagosomal and cytosolic phases and that phosphoinositide metabolism is essential for cytosolic proliferation of *F. tularensis*. Connor et al. [14] revealed that 71 host proteins are required for intracellular survival of *Y. pestis*. Of particular, interest was the enrichment for genes involved in endosome recycling.

Using the gene trapping approach, Carette et al. [76] first identified several host factors that are required for EBOV infection. These include a cholesterol transporter Niemann–Pick C1 factor involved in the fusion of endosomes and lysosomes (homotypic fusion and protein sorting complex), biogenesis of endosomes (PIKFYVE), lysosomes (BLOC1S1, BLOC1S2), and targeting of luminal cargo to the endocytic pathway [76]. Many of these hits reoccurred in several CRISPR and small interfering RNA (siRNA)/shRNA screenings [77,78,81]. In addition, lysosomal protein (BRI3) and a GTPase involved in the regulation of vesicle trafficking (RAB39B), PI3K, calcium/calmodulin kinase-related network, and de novo pyrimidine synthesis pathway are essential for EBOV replication and transcription [78–80]. The application of RNAi screening has been utilized for other viral pathogens such as henipavirus, JUNV, poxvirus, *Vaccinia virus*, and VEEV [75,82–86]. It is demonstrated that catalytic activity of fibrillarin, the enzymatic subunit of the snoRNP complex that is responsible for catalyzing the transfer of a methyl donor from a bound cofactor *S*-adenosyl methionine to ribose sugars of the target pre-rRNA, is required for henipavirus infection [82]. Voltage-gated calcium channel (VGCC) subunits were shown to be important in JUNV–cell fusion and entry into cells. Gabapentin, an FDA approved anticonvulsant drug against $\alpha_2\delta_2$ subunit-containing VGCCs, inhibited replication of the vaccine strain of JUNV in mice [75]. Other siRNA-based screens against *V. virus* identified that AMP-activated protein kinase (AMPK) promotes viral entry through the control of actin dynamics, and knockdown of nuclear pore protein (Nup62) arrests virion morphogenesis [83–85]. Lastly, an siRNA screen identified trafficking host factors that modulate VEEV infection [86].

An alternative approach to gain an in-depth understanding of host–pathogen interactions during infection is to construct a protein–protein interaction network between host protein and bacterial virulence factors. Using a yeast two-hybrid (Y2H) library, Memišević et al. [16] identified a molecular network that governs *B. mallei* infection. Similarly, a Y2H study conducted by Yang et al. [87] showed the involvement of focal adhesion, regulation of cytoskeleton, leukocyte transendoepithelial migration, Toll-like receptor (TLR), and MAPK signaling pathways during *Y. pestis* infection. To complement the Y2H study, reverse-phase protein microarray analysis was used to interrogate changes in protein expression and posttranslational modification. This further revealed the roles of AMPK-α1, Src, and GSK3β in regulating *B. mallei* and *B. pseudomallei* infection [88], and thus, as viable host targets for countermeasure development.

4 Development of countermeasures to biothreat agents

Prior to the initiation of medical countermeasure development against specific pathogens, a target product profile (TPP) is needed to define the required features of potential drug candidates (e.g., route of administration, prophylactic vs therapeutic, trigger to treat, and onset of action requirements). Once a TPP is in place, a screening funnel is drafted that sets laboratory criteria and defines clear go/no-go decision points that are needed to progress countermeasures from discovery through preclinical development and into human clinical trials. Irrespective of the types of assays used for countermeasure screening, compounds identified as having significant inhibition in primary screens are validated in subsequent dose–response experiments to determine the half maximal effective concentration (EC_{50}) and cytotoxic concentration (CC_{50}). Potent compounds that have an adequate selectivity index (e.g., >10) that is defined as a ratio of CC_{50}/EC_{50}, are then often tested in orthogonal assays in appropriate cells/tissues to better understand or validate the antipathogen activity. Ideally, compounds are further optimized for potency, selectivity, physicochemical, and pharmacokinetic (PK) properties and safety prior to in vivo evaluation to assess efficacy in appropriate animal models of infection (Fig. 7.4).

Many of the therapeutics that are in different stages of either preclinical or clinical development for select biothreat pathogens include small molecule antivirals (Tables 7.3 and 7.4), antibody (or antibody cocktails) against viruses or bacteria/virulence factors (Table 7.5), and combination drug therapy (Table 7.6). The increased use of antivirals and antibiotics has set the stage for rapid adaptation mechanisms that microbes can use to counteract them. The development of antimicrobial resistance is one of the biggest public health threats and hence alternative approaches to treat infectious diseases are urgently needed. Table 7.7 lists the resistance mechanisms identified in each biothreat bacterial pathogen and provides references for targets of resistance. Since stand-alone antibiotics may not be sufficient to overcome resistance and/or completely clear some biothreat bacterial infections, we have also included encouraging data on host directed therapeutics, and combination therapy.

4.1 Host-directed therapy

HDT is an emerging approach in the field of antiinfectives discovery. The strategies behind HDT can include modulation of host immune responses, or interference/manipulation/targeting of host-cell factors that are required for pathogen replication [202]. For example, in a potential bioterror scenario, where the identity of the etiological agent causing the disease is unknown, stimulation of innate immunity may be particularly useful as induced immune responses are often capable of providing protection against a broad range of pathogens. Although no FDA-approved HDT therapies are yet available for treating infectious diseases, we have summarized in this section the antimicrobial

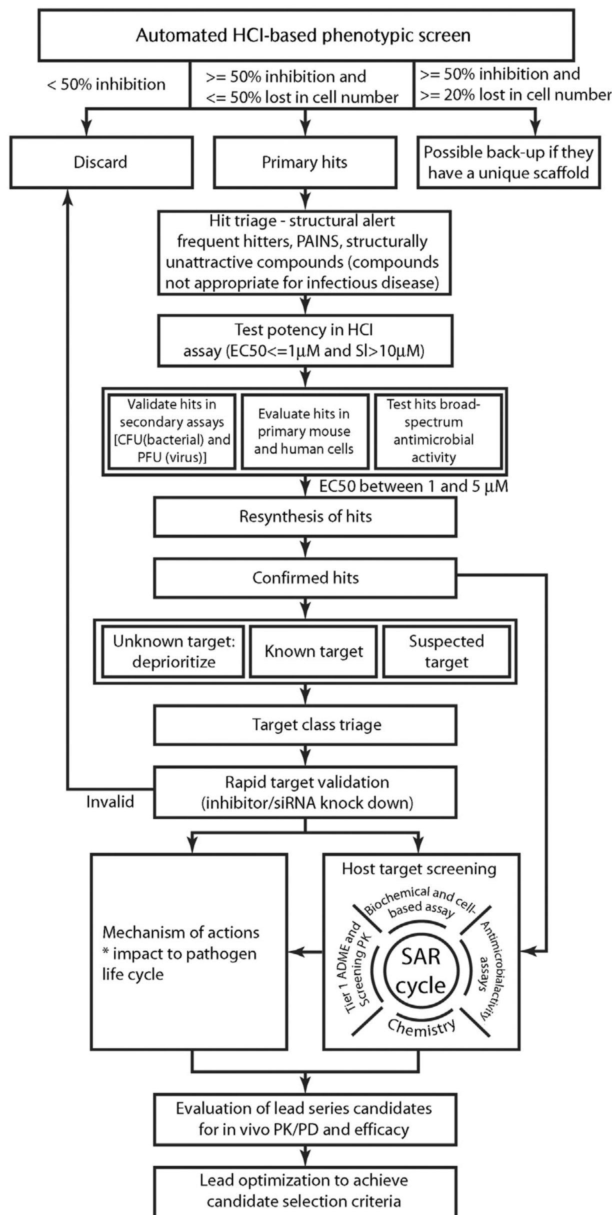

Figure 7.4 ***Screening funnel to identify small molecule therapeutics.*** Primary screening of small molecule chemical libraries in the phenotypic HCI assay will identify compounds that inhibit pathogen infection as well as those that may contribute to cellular toxicity. Generally, hits that show ≥50% infection inhibition and ≤20% loss in cell number are then subjected to hit triage or in silico filtering wherein compounds with optimal physicochemical properties such as solubility, Lipinski's Rule of 5, metabolism are selected for potency testing in the phenotypic screening assays. Compounds that exhibit an $EC_{50} \leq 1$ μM and SI, which is a ratio of $CC_{50}/EC_{50} > 10$, are then further optimized through iterative cycles of synthesis, testing in cell-based and orthogonal assays and in in vitro ADMET studies to improve potency and physicochemical properties. If the target of the hit molecule is identified then a target-based screen is performed and the hits identified are optimized through the iterative structure–activity relationship cycle. The Lead series candidates are then evaluated in vivo for their pharmacokinetic properties and then for efficacy in appropriate challenge models of infection. *ADMET*, absorption, distribution, metabolism, excretion, and toxicity; *HCI*, high-content imaging; *SI*, selectivity index.

Table 7.3 In vitro and in vivo PK, tolerability, and efficacy studies of antivirals against biothreat viral agents.

Therapeutic	Target	Efficacy data	PK and tolerability	Additional information and comments	References
EBOV					
BCX4430	Viral polymerase	In vitro—Kikwit EC_{50}: 11.8 μM; EC_{90}: 25.4 μM In vivo—mice: 150 mg/kg BID PO; 90% survival; 150 mg/kg BID IM; 100% survival NHP: 25 mg/kg BID D0–14; 100% survival; 100 mg/kg BID IM D2, then 25 mg/kg BID D3–11, 100% survival (67% with same regimen starting on D3) In vivo—HP: 10 mg/kg iv D3–15; 100% protection	Well tolerated in all efficacy studies. Very short plasma half-life in mouse and NHP ($T_{1/2}$ = 2–10 min). Mouse, 150 mg/kg, IM: liver, C_{max} (triphosphate) = 65 μM, T_{max} = 8 h, $T_{1/2}$ = 4.3 h. Conversion to triphosphate in hepatocytes: mouse>human ~NHP	BCX4430-TP levels in mouse liver at 150 mg/kg IM are ~2.5× above EC_{90} value. Distribution into other tissues/cells not reported	[89–91]
Favipiravir (T-705)	Viral polymerase	In vitro—IC_{50}: 67 μM; EC_{50} = 281 μM (Kikwit), 223 μM (Makona), 51 μM (Marburg Ci67)	Favipirivir-ribose-TP concentrations were measured in various cell lines and mouse tissues	Intracellular ribosylation required prior to triphosphorylation to active drug	[92–96]
		In vivo—mice: 100% survival at 300 mg/kg D0–7 against aerosol challenge; 100% survival at 300 mg/kg beginning D6 following intranasal challenge [92]; 90% survival observed at 8 mg/kg [41]. NHP: Marburg (Angola), 83% survival at 250 mg/kg, iv loading dose + 150 mg/kg, BID for 13 days	Biodistribution in mice assessed by PET imaging. Plasma favipirivir-ribose-TP levels in NHPs following 200 mg/kg, iv loading dose and 150 mg/kg, iv, BID daily dose were 4–34 μM—below the MARV EC_{50} of 51 μM		

(*Continued*)

Table 7.3 In vitro and in vivo PK, tolerability, and efficacy studies of antivirals against biothreat viral agents. *(Cont.)*

Therapeutic	Target	Efficacy data	PK and tolerability	Additional information and comments	References
GS-5734	Viral polymerase	In vitro—replication: EC_{50}: 0.086–0.14 μM	NHP PK 10 mg/kg, iv, short plasma $T_{1/2}$ of GS-5734, rapid intracellular conversion to triphosphate with persistent levels >EBOV EC_{50} for 24 h, intracellular triphosphate $T_{1/2}$ = 14 h	NHP tissue distribution plasma~testes> eye>brain	[97]
Clomiphene	CAD, entry inhibitor	In vitro—IC_{50}: 11.1 μM (Kikwit) IC_{50}: 3.83 μM (Mayinga) In vivo—Mice: 60 mg/kg IP QD on days 0, 1, 3, 5, 7, and 9; 90% survival	Mouse PK unavailable. Human PK 50 mg QD: C_{max} = 37 nM. Protein binding not reported, but likely high due to structure similarity to toremifene	Estrogen receptor modulator, human-free drug exposure = Ebola EC_{50}	[98,99]
Bepridil	CAD, entry inhibitor	In vitro—IC_{50}: 5.08 μM (Vero) IC_{50}: 3.21 μM (HepG2) In vivo—Mice: 12 mg/kg; 100% survival	Mouse PK unavailable; human PK 300 mg PO QD: C_{max} ~ 6.3 μM, PPB > 99%	Calcium channel blocker, human-free drug plasma exposure = Ebola EC_{50}, QT prolongation issues	[100]
Brincidofovir	Unknown	In vitro—EC_{50}: 120 nm–1.3 μM In vivo—no preclinical efficacy reported		Interferes with viral DNA replication	[101,102]
Type I IFN	NA	In vitro—IFN-α IC_{50}: 0.038 μM IFN-β IC_{50}: 0.016 μM In vivo—IFN-α2b in NHP: delayed time to death IFN-β in NHP: 10.5 μg/kg at 18 h and days 1, 3, 5, 7, and 9; delayed time to death			[103–105]

TKM-100802, TKM-130803	L,VP35,VP24	In vitro—not available In vivo—NHP: 100% survival against Kikwit and Makona		Lipid nanoparticle formulation of siRNAs	[106,107]
AVI-6002	VP24/VP35	In vitro—not available In vivo—NHP: up to 63% survival at 40 mg/kg		Combination of AVI-7537 and AVI-7539 phosphorodiamidate morpholino oligomers	[108–110]
AVI-7537	VP24	In vitro—0.585 μM In vivo—NHP: 40 mg/kg; 75% survival		Phosphorodiamidate morpholino oligomer	[108,110]
LASV					
Ribavirin	Viral polymerase	In vitro—yield reduction assay, Vero cells, IC_{90} = 2.5 μM, Junin (Romero); 3.6 μM, Junin (Candid#1); plaque assay, Junin (Romero), Vero E6 cells, EC_{90} = 71 μM In vivo—NHP: 50 mg/kg, s.c. (loading dose) followed by 10 mg/kg, s.c. every 8 h through day 18; 4 animals dosed immediately after infection, 4 dosed 5 days post infection; 8/8 survived versus 4/10 in control group	Ribavirin is intracellularly triphosphorylated to the active drug. In humans, plasma concentrations of ribavirin have been correlated to virological response	Nucleoside drug; FDA approved for RSV and HCV	[111–114]

(*Continued*)

Table 7.3 In vitro and in vivo PK, tolerability, and efficacy studies of antivirals against biothreat viral agents. *(Cont.)*

Therapeutic	Target	Efficacy data	PK and tolerability	Additional information and comments	References
Stampidine	Viral polymerase	In vivo—CBA mice, cerebral injection of Lassa (Josiah): 50 mg/kg, i.p., dosed daily for 6 days starting 24 h prior to infection, 90% survival compared to 25% vehicle survival	Rapidly converted to its active form (Ala-MP) within 5 min in plasma. No notable toxicity after daily i.p. or p.o. admin for 8 weeks (cumulative dose 6.4 g/kg)	Nucleoside monophosphate prodrug; reverse transcriptase inhibitor against HIV	[115]
Zidampidine	Viral polymerase	In vivo—CBA mice, cerebral injection of Lassa (Josiah): 25 mg/kg, i.p., dosed daily for 6 days starting 24 h prior to infection, 100% survival compared to 28% vehicle survival	Rapidly converted to its metabolites Ala-AZT-MP and AZT following iv injection. Nontoxic to mice up to 250 mg/kg	Nucleoside monophosphate prodrug of zidovudine (AZT)	[116]
LASV, JUNV					
LHF-535	Entry inhibitor	In vitro—yield reduction assay, Vero cells, IC_{90} = 9.3 nM, Junin (Romero); 3 μM, Junin (Candid#1) In vivo—Tacaribe virus, AG129 mice: 100% survival, 10 mg/kg/day, p.o. for 14 days starting 30 min prior to infect.; 60%–90% survival when dosed 24–72 h post infection	Not available	Small molecule	[111]
Favipiravir (T-705)	Viral polymerase	In vitro—plaque assay, Junin (Romero), Vero E6 cells, EC_{90} = 21 μM In vivo—LASV (Josiah), cynomolgus macaques: 4/4 survival versus 0/4 placebo, 300 mg/kg, iv on day 4 followed by 300 mg/kg, s.c., qd days 5–17	PK, biodistribution, and favipiravir-ribose-triphosphate levels discussed above	Intracellular ribosylation required prior to triphosphorylation to active drug	[93–95, 112, 117]

VEEV

β-D-N4-hydroxycytidine	Viral polymerase	In vitro—VEEV EC_{50} = 1.2 μM (HeLa), 1.3 μM (Vero); 99% inhibition at 10 μM (astrocytes)	Rat brain nucleoside TP levels after single 50 mg/kg, p.o. dose are near VEEV EC_{50} values (526 ng/g at 2.5 h, 135 ng/g at 24 h)	Broad-spectrum antiviral (EEEV, WEEV, VEEV, and numerous nonbiothreat viruses such as CHIKV, MERV, influenza, and RSV)	[118]
ML366	nsP2, nsP4	In vitro—EC_{50} (VEEV TC-83) = 32 nM (Vero) In vivo—VEEV TrD (s.c. injection), BALB/c mice, 12.5 mg/kg, i.p. every 12 h for 8 days starting 2 h preinfection, 100% survival	Mouse brain drug levels (single 10 mg/kg, i.p.) at 2 h post dose were 0.35 μM		[119,120]
BDGR-4	nsP2, nsP4	In vitro—EC_{50} (Vero) = 47 nM (VEEV TC-83), 150 nM (EEEV), 102 nM (WEEV) In vivo—VEEV TrD (s.c. injection), BALB/c mice, 12.5 mg/kg, i.p. every 12 h for 8 days starting 24 h post infection, 100% survival (90% starting 48 h post infection). EEEV (s.c. injection), C57BL/6 mice, 25 mg/kg, i.p. every 12 h for 8 days starting 2 h preinfection, 90% survival		Close-in analog to ML366	[119]

EBOV, Ebola virus; *HCV*, Hepatitis C virus; *IFN*, Interferon; *JUNV*, Junin virus; *LASV*, *Lassa virus*; *NHP*, nonhuman primate; *NHP*, nonhuman primate; *PPB*, plasma protein binding; *PK*, pharmacokinetic; *PET*, positron emission tomography; *RSV*, respiratory syncytial virus; *TP*, triphosphate; *VEEV*, venezuelan equine encephalitis virus.

Table 7.4 Antiviral therapeutics (small molecules, antibodies, and protein) that have been evaluated in humans.

IND	Clinical trial phase	Results/Status	Other clinical data	References
EBOV				
BCX4430	Phase I (NCT02319772)	Phase I complete; results not available yet	N/A	
Brincidofovir	Phase II (NCT02271347)	Terminated due to low enrollment; not currently under further development as EBOV therapeutic	Administered to 5 patients during the outbreak, often in combination with other therapies	[101,121,122]
GS-5734	Phase II (NCT02818582)	Phase I complete; Phase II for efficacy in survivors with viral persistence in semen	Administered to a newborn in combination with ZMapp and buffy coat transfusion; patient survived	[123]
TKM-100802	Phase I (NCT02041715)	Terminated	100802: Administered to two patients in combination with convalescent plasma; both survived	[124,125]
TKM-130803	Phase II (PACTR201501000997429)	Terminated early; did not demonstrate efficacy; development has been suspended		
Favipiravir (T-705)	Phase II (NCT02329054: JIKI; NCT02662855: Sierra Leone)	Efficacy in patients with low-to-moderate levels of virus (C_t values >20)	Administered with ZMab to a patient who recovered; administered to a patient with convalescent plasma who recovered; retrospective study indicated increased survival and lower viral loads	[126–129]
ZMapp	Phase II (NCT02363322)	Inconclusive efficacy due to insufficient statistical power	Administered to patients during 2014 outbreak, often in combination with other therapies	[130]

AVI-6002, AVI-7537	Phase I (AVI-6002: NCT01353027; AVI-7537: NCT01593072)	6002: Favorable safety and tolerability 7537: Terminated prior to enrollment; further development has been suspended	N/A	
IFN-β	Phase I/II (ISRCTN17414946)	Results not yet released	N/A	
LASV				
Ribavirin	Phase II	Evidence of efficacy in patients with ASTs > 150 IU/L: iv treatment (16 mg/kg q6h for 4 days, then 8 mg/kg q8h for 6 days; serum levels 12–100 μM) starting within 6 days of fever onset, 1/20 fatality (11/43 if >7 days after fever onset); p.o. treatment had 1/5 fatality (<6 days) and 1/9 (>7 days); placebo had 11/18 fatality (<6 days) and 22/43 (>7 days)	FDA approved for RSV and HCV with black box warnings for birth defects and breakdown of red blood cells. Used off label to treat Lassa fever. Need for higher powered randomized trials, inclusion of comparator and patients at a later stage of disease	[131]

AST, Aspartate Aminotransferase; *EBOV*, Ebola virus; *FDA*, Food and Drug Administration; *HCV*, Hepatitis C virus; *IND*, investigational new drug; *LASV*, *Lassa virus; RSV*, Respiratory syncytial virus.

Table 7.5 Single or cocktail of different antibodies and reported efficacy studies in animal models or humans.

Biothreat agent	Therapeutic target of antibody	Efficacy data	References
Bacterial			
Bacillus anthracis	PA, LF, and EF, and the capsule	Details of the mAbs developed against *B. anthracis* can be found elsewhere	[132]
Burkholderia mallei and *Burkholderia pseudomallei*	Capsular PS or the LPS	CPS individually, or in combination with an LPS mAb, prevents *B. pseudomallei* infection in mice. Although both mAbs confer protection when given singly, the combination treatment provided significantly better protection at low doses	[133]
Coxiella burnetii	Phase I LPS	1E4 is an antibody targeting PI-LPS of Cb. Fab1E4 (Fab fragment of 1E4), recombinant murine single-chain variable fragment (muscFv1E4), and a humanized single-chain variable fragment (huscFv1E4) were able to inhibit *C. burnetii* infection in mice but that their ability to inhibit *C. burnetii* infection was lower than that of 1E4	[134]
Francisella tularensis	LPS	mAbs against the LPS of *F. tularensis* LVS could be successfully used to treat LVS-induced pneumonia but not a *F. tularensis* type A strain. Anti-LVS antibodies failed to protect mice challenged with *F. tularensis* Schu S4	[21]
Yersinia pestis	Capsid F1 protein and the Lcr V-protein	A synergistic effect was observed when anti-F1-specific human mAb (m252) and two anti-V-specific human mAbs (m253, m254) were combined. Incomplete to complete protection was achieved when m252 was given at different times post challenge	[22]
Viral			
Arenaviridae JUNV	Glycoprotein	Human case reports: convalescent serum therapy controls active infection [135] Guinea Pig: neutralizing antibodies generated by vaccination or monoclonal antibody cocktails are protective	[136,137]
Arenaviridae Lassa	Glycoprotein	Human case reports: convalescent plasma therapy–mixed success at controlling infection Guinea Pig: presence of neutralizing GP antibodies can control infection NHP: cocktail of 5 human mAbs conferred high level of protection	[138,139]
Nanoviridae CCFH	Unknown	Human case reports: the use of hyperimmunoglobulin obtained from survivor plasma showed modest success	[140–142]

Filoviridae EBOV	Glycoprotein (Z-EBOV)	Human clinical trials: ZMapp, c2G4, c4G7, and c13C6 combination cocktail currently being evaluated for safety and efficacy in West Africa and the United States; nine EBOV-infected individuals were administered ZMapp under compassionate use, determined the treatment course to 3 doses at 50 mg/kg at 3 intervals post infection; decreased viral load was observed in all patients (NCT02363322)	[130,143, 144]
Paramyxoviridae Nipah	F and G envelope glycoproteins	Hamster: IP injection of large amounts of serum from donors receiving antiserum pools of nonspecific neutralizing antibodies can protect against lethal disease Passive administration of murine mAbs against NiV F and G conferred protection	[145–147]
Poxviridae Monkeypox	Mature virion and extracellular virion	NHP: prophylactic antibody treatment can protect against severe monkeypox disease in marmoset model	[148]
Poxviridae Variola	Multiple MV, H3, A27, D8, and L1, EV B5, and A33	Human case reports: antibodies generated from *Vaccinia virus* can protect against VAR infection Mice: superior in vivo protection against VACV infection was achieved by administration of a mixture of human mAbs that targeted multiple viral antigens	[149–151]
Togaviridae Chikungunya	Glycoprotein E2 subunit	In vitro: human mAbs 5F10 and 8B10 were isolated from patients infected with CHIKV and found to have neutralizing properties Mice: 4J21 and 5M16 found to be protective; MAb 152 protected mice against was highly effective postexposure treatment of CHIKV infection; MAb 102 and 152 or MAb 152 and 166 cocktails were shown to confer protection in mice exposed to lethal doses of CHIKV and enhanced the window of treatment, as compared to MAb 152 therapy alone NHP: combination use of MAbs 152 and 166 in rhesus macaques noted reduced viral spread and infection with viral RNA persistence	[152–154]

CCFH, Crimean-Congo hemorrhagic fever; *CPS*, capsular polysaccharide; *EF*, edema factor; *GP*, glycoproteins; *IP*, Intraperitoneal; *JUNV*, Junin virus; *Lcr*, low-calcium response; *LF*, lethal factor; *LPS*, lipopolysaccharides; *LVS*, live vaccine strain; *mAb*, monoclonal antibody; *NiV*, Nipah virus; *NHP*, nonhuman primate; *PA*, protective antigen; *PS*, polysaccharides; *VACV*, Vaccinia virus; *VAR*, Varicella.

Table 7.6 Combination therapies for biothreat bacterial pathogens.

Combination therapeutics		Notes	References
Bacillus anthracis			
Antibiotics	AIGIV	Combination therapy with antibiotics and AIGIV is more effective than antibiotics alone in a rabbit model of inhalational anthrax and improved survival compared to the antibiotic treatment alone	[155]
Ciprofloxacin	Clindamycin	Treatment of rabbits with systemic anthrax with clindamycin and ciprofloxacin had improved efficacy compared to monotherapy and could be used to prevent relapse of infection	[156]
Ciprofloxacin	PA IgG antibodies	Combination therapy for anthrax, including antiprotective antigen (PA IgG) antibodies and ciprofloxacin in a rodent anthrax model increased survival significantly compared to ciprofloxacin treatment alone	[157]
Levofloxacin	Raxibacumab	Combination therapy with raxibacumab, an IgG1 monoclonal antibody that binds protective antigen, and the antibiotic levofloxacin provides protection in rabbits late in the disease course	[158]
Oligochlorophen	Antibiotics	Targeting cytoskeletal proteins such as FtsZ with oligochlorophen analogs is a promising new treatment method that has a 10-fold lower development of resistance compared to antibiotics used for anthrax treatment in humans	[159]
Penicillin, meropenem, or rifampin	Linezolid	Treatment of antibiotic-resistant inhalation anthrax with linezolid and penicillin, meropenem, or rifampin had the inhibitory effect on mean lethal factor levels compared to the control groups and successfully treated fluoroquinolone-resistant *B. anthracis* infection	[160]
Rifampin	Clindamycin	Combination therapy for anthrax with rifampin and clindamycin was shown to be synergistic in vitro	[161]

Table 7.6 Combination therapies for biothreat bacterial pathogens. *(Cont.)*

Combination therapeutics		Notes	References
***Brucella* spp.**			
Doxycycline	Rifampin	Successful combination therapies used to treat pulmonary brucellosis in humans is doxycycline and rifampin for 6 weeks	[162]
Burkholderia mallei			
Antibiotic	Heat-killed vaccine	Combination of an antibiotic moxifloxacin, azithromycin, or sulfamethoxazole-trimethoprim, and vaccination using heat-killed *B. mallei* can protect BALB/c mice from lethal glanders infection, potentially by stimulating immune responses, such as gamma interferon, which acts synergistically with antibiotic therapy to inhibit bacterial growth	[163]
Enrofloxacin, trimethoprim, and sulfadiazine	Doxycycline	Successful 12-week combination treatment of parenteral administration of enrofloxacin and trimethoprim with sulfadiazine followed by oral administration of doxycycline eliminated *B. mallei* from glanderous horses during an outbreak	[164]
Burkholderia pseudomallei			
Antibiotics	Farnesol	Combination therapy with farnesol a sesquiterpene alcohol that damages biofilm matrix and interferes with cell wall and peptidoglycan biosynthesis, facilitates antimicrobial penetration, and reduces the minimum biofilm eradication concentration for ceftazidime, amoxicillin, doxycycline, and sulfamethoxazole-trimethoprim in vitro	[165,166]
Ceftazidime	Avibactam	Avibactam restores susceptibility to ceftazidime for genetically diverse extremely drug resistant isolates of *Burkholderia* from cystic fibrosis patients by binding PenA and the combination treatment significantly improved survival of larvae infected with the drug resistant isolates	[166]

(*Continued*)

Table 7.6 Combination therapies for biothreat bacterial pathogens. *(Cont.)*

Combination therapeutics		Notes	References
Ceftazidime	IFN-γ	Interferon gamma–induced reactive oxygen species with ceftazidime leads to synergistic killing of intracellular *B. pseudomallei* and markedly increases the effectiveness of antimicrobial therapy for the treatment of *B. pseudomallei* infection in mice	[167,168]
Clostridium botulinum			
Antibody cocktail		BoNT serotypes and subtypes differences present a significant challenge for creating monoclonal antibody treatments for neutralization, by diversifying the V-regions of mAbs and selecting cross reactivity, a combination treatment of three antibodies neutralized BoNT/F1, F2, F4, and F7 in mice and was 150 times more potent than equine antitoxin	[169]
Coxiella burnetii			
Doxycycline	Chloroquine	Combination therapy of doxycycline and hydroxychloroquine combination shortened the duration of therapy and reduced the number of relapses in patients with Q fever endocarditis. And a case of Q fever endocarditis with biological prosthetic aortic valve and aortic homograft was successfully treated with doxycycline and chloroquine combination therapy	[170,171]
Francisella tularensis			
Antibiotics	Uptake inhibitors and inflammatory inhibitors	Cytochalasin B, LY294002, wortmannin, nocodazole, MG132, and XVA143 inhibitors reduce *F. tularensis* update and reduce inflammatory cytokine production and can be used in combination with antibiotics to improve survival of infected mice	[172]
Gentamicin	Membrane antigen immunization	Postexposure immunization with membrane protein fraction antigens and treatment with low-dose gentamicin increased survival of mice and significantly reduced bacterial burdens in the liver and spleen	[173]

Table 7.6 Combination therapies for biothreat bacterial pathogens. *(Cont.)*

Combination therapeutics		Notes	References
Yersinia pestis			
Antibiotics	Efflux pump inhibitor	Combination therapy, including antibiotics with an efflux pump inhibitor, would be a novel mechanism to restore the efficacy of the antibiotic in resistant strains of *Y. pestis*	[174]
Antibody therapy	Corticosteroid	The addition of antiinflammatory methylprednisolone, a corticosteroid, in combination with antibody therapy correlates with improved mouse survival, with reduction in neutrophil and matrix metalloproteinase 9 in the tissue, and the mitigation of tissue damage	[175]
Ciprofloxacin	L-97-1	A novel postexposure medical countermeasure L-97-1, an A_1 adenosine receptor antagonist blocks LPS-induced activation of immunomodulatory cytotoxic substance accumulation to prevent acute lung injury, and in combination with ciprofloxacin improves survival of rats following infection with *Y. pestis*	[176]

AIGIV, anthrax immune globulin intravenous; *IFN*, interferon; *LPS*, lipopolysaccharide; *PA*, protective antigen.

potential of several small molecule immunomodulators and host cell factors that have been investigated to date.

Immunomodulators directly target the host rather than the pathogen (Fig. 7.5). This is accomplished by targeting pattern recognition receptors, such as TLRs that are present on innate immune cells in the host to detect features of microbes known as pathogen-associated molecular patterns. Since immunomodulators target host immune cells, they are an attractive candidate for use against bacterial agents as they are unlikely to result in the development of antibiotic resistance even after repeated use. In particular, the threat of an intentional release of a highly virulent bacterial pathogen that is either intrinsically resistant to antibiotics, or has been weaponized via the introduction of antibiotic resistance, makes immunomodulation an attractive complementary or alternative strategy to directly targeting bacterial biothreat agents. For example, a synthetic TLR9 agonist, *5'-C-phosphate-G-3'* oligodeoxynucleotide (CpG ODN), appears to be able to stimulate protective immunity against intracellular bacterial infection and/or eliminate chronic infections. Indeed, studies in mice have demonstrated that the innate immune defenses activated by CpG ODNs protect against lethal challenge with *B. anthracis*, *B. mallei*, and

Table 7.7 Antibiotic resistance mechanisms for biothreat bacterial pathogens.

Antibiotics	Intrinsic and targeted resistance genes or proteins	References
Bacillus anthracis		
β Lactams	*bla1* (penicillinase), *bla2* (cephalosporinase)	[177]
Folate synthesis (trimethoprim)	Intrinsic dihydrofolate reductase resistance	[178]
Macrolides	*ermJ*	[179]
Quinolones	*gyrA*[a], *gyrB, glrA, glrB, parC*, and/or *parE* genes	[180,181]
***Brucella* spp.**		
β Lactams	RND-type efflux pumps	[182]
Polymyxin	Phospholipase A1 esterase	[183]
Quinolones	*gyrA*[a], *gyrB, pare*, and RND-type efflux pumps	[182,184, 185]
Tetracyclines	RND-type efflux pumps	[182]
Burkholderia pseudomallei		
Aminoglycosides	RND-type efflux pumps, *S*-adenosyl-L-methionine-dependent methyltransferase, *amrR*	[186,187]
β Lactams	*penA*[a], *nlpD1, dacC, FlgN, sch, TR70_0856, TR70_1911, ftsI, amrR*, *bpeR*, *bpeT*, *spoT*, tRNA, rRNA, proteins with unknown function, SerS seryl-tRNA synthetase, and RND efflux pump AmrAB-OprA, and BpeEF-OprC	[186, 188–192]
Macrolides	AmrAB-OprA efflux pump	[193]
Quinolones	AmrAB-OprA efflux pump, BpeAB-OprB efflux pump	[194]
Sulfamethoxazole/ Trimethoprim	RND BpeEF-OprC efflux pump, LysR-type regulator BpeT BpeS, Ptr1, FolA, AmrR TetR-type regulator, AmrAB-OprA, *met*F	[186,195]
Coxiella burnetii		
Quinolones	*gyrA*[a]	[196]
Tetracyclines	Putative protein secretion targets, biosynthesis of pantothenate and coenzyme A, aspartate biosynthesis, DNA replication	[197]
Francisella tularensis		
β Lactams	*blaB1*	[198]
Chloramphenicol	23S rRNA, the L4 and L22 ribosomal proteins, and overexpression of efflux pumps	[199]
Quinolones	*gyrA*[a] and *gyrB*	[200]
Yersinia pestis		
Aminoglycosides	AcrAB-TolC efflux pump	[174]
Macrolides	AcrAB-TolC efflux pump	[174]
Quinolones	*gyrA*[a], *gyrB, and parC*	[201]
Rifampin	AcrAB-TolC efflux pump	[174]

[a] Target of antibiotic.

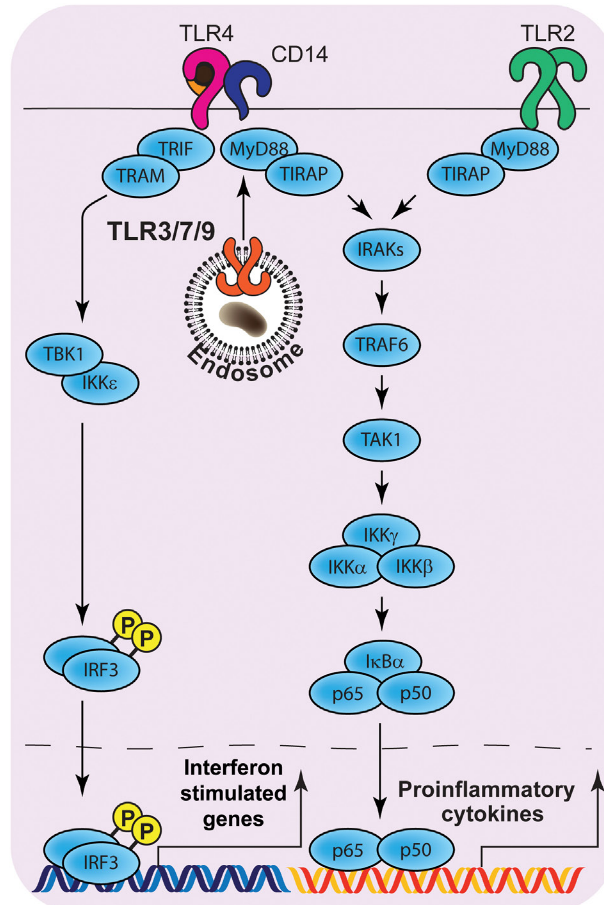

Figure 7.5 ***Potentiating immune response by activating TLR signaling pathways.*** With the exception of TLR3, all TLRs activate MyD88 dependent pathway which leads to NF-κB mediated proinflammatory cytokine upregulation. TLR3 and TLR4 can activate TRIF-dependent pathway which leads to IRF3 mediated interferon stimulated gene upregulation. TLR2 and TLR4 reside on the cell membrane whereas TLR3/7/9 are localized in the endosomal compartment. *TLR*, Toll-like receptor.

F. tularensis or their surrogates [203]. Similarly, human monocyte-derived macrophages treated with poly(I:C), a synthetic TLR3 agonist, showed significantly reduced intracellular *F. tularensis* [both Schu 4 and LVS (live vaccine strains)] replication. Mice administered with poly(I:C) before or after Schu 4 or LVS infection showed reduced bacterial burden in the lungs and prolonged survival. Mice treated with poly(I:C), challenged with *F. tularensis*, and then treated with levofloxacin showed 100% survival relative to no survival in animals receiving levofloxacin alone [204].

In addition to targeting innate immune cell receptors, there is a growing interest in modulating autophagy as an immunotherapeutic intervention. Autophagy is a dynamic

process that targets cellular cytoplasmic contents for lysosomal degradation. More specifically, xenophagy is a type of selective autophagy that specifically targets intracellular pathogens to lysosomes, retracing their replication and survival [205]. The use of autophagy inducer rapamycin, decreased the survival of *B. pseudomallei* in vitro [206]. However, several bacteria exploit autophagic machinery as part of their intracellular life cycles (i.e., *Brucella abortus*, *C. burnetii*, and *F. tularensis*). Therefore infection may be exacerbated by the induction of autophagy (Fig. 7.1C) [205]. Research to further understand the balance between infective and protective cellular targets in the autophagy pathway may enhance its utilization as a therapeutic target.

HTS of FDA-approved drugs is another approach to identifying compounds that were previously approved for other disease indications but may have the potential to be repurposed as antiinfectives. Trifluoperazine (an antipsychotic), amoxapine (an antidepressant), and doxapram (a breathing stimulant) mitigated fatal *Y. pestis* infection in a pneumonic plague murine model [207]. At 48 h postinfection, these drugs provided animals with up to 100% protection against challenge with bubonic or pneumonic plague agents when administered in combination with levofloxacin [208]. Multiple FDA-approved drugs targeting G-protein coupled receptors and calcium fluxes inhibited *C. burnetii* and *B. abortus*, whereas drugs targeting cholesterol traffic attenuated *C. burnetii* [209]. Similarly, increasing evidence suggested statin, a 3-hydroxy-3-methylglutaryl-coenzyme-A reductase inhibitor, possesses antibacterial activity by the inhibition of sterols, prenylation, and isoprenoids (*C. burnetii*), the inhibition of antiinflammatory cytokines (*Y. pestis*), and the modulation of phagosome maturation (*C. burnetii*) [210]. It was demonstrated that a low dose of Gleevec, an anticancer drug inhibiting Abl1, c-Kit, and related protein tyrosine kinases, can increase the number of myeloid cells in the bone marrow, blood, and spleen and enhance antimicrobial responses in a mouse model of *F. tularensis* infection [211].

In the case of viruses, small molecule targeting of innate immune receptors has also shown efficacy in several relevant viral models of infection. For example, treatment with poly(IC:LC) has also been protective against EBOV infection in NHPs [212]. Prophylactic pulmonary administration of TLR7 ligand (TMX201) significantly protected mice from lethal infection with VEEV [213]. TLR3 and TLR9 agonists have also been shown to improve the efficacy of postexposure therapeutics against smallpox [214]. Sometimes modulation of host pathophysiological responses can be evaluated as a target. Hemorrhagic fever virus pathophysiology includes the stimulation of procoagulant pathways and increased permeability of the vascular endothelium; therefore these processes are being evaluated as possible targets for therapeutic intervention. This could be accomplished by utilization of an anticoagulant, such as recombinant nematode anticoagulant protein c2 (rNAPc2) that blocks initiation of the extrinsic coagulation pathway by inhibiting the tissue factor–factor VIIa complex [215,216]. rNAPc2 has been shown to be highly protective in macaques infected with a lethal dose of Ebola Zaire virus, when treatment was initiated 1 day post viral challenge [216,217].

4.2 Antibody therapy

Whereas HDT targets the host directly, antibody therapy is the passive process of activating the immune system to respond to microbial threats. Sources of antibodies can include individuals that survive infection or have received a prophylactic vaccine against a microbe. Alternatively, antibodies can also be generated ex vivo using cell culture. Historically, antibody-based serum or plasma therapy has been widely used to treat a variety of infectious diseases. Limitations for clinical use arise however from the polyclonal nature of serum antibodies, resulting in lot-to-lot variation, approaches for determination of correct dose levels and regimens, and a risk for allergic reactions and transmission of transfusion-borne diseases.

In general, limited clinical applications for antibody therapy existed until the development of technology that allowed the production of mAbs through the use of hybridomas [218]. Hybridomas allow for the production of homogenous antibodies with the same specificity of a single immunoglobulin class and isotype. Further advancements made it possible to humanize or generate fully human mAbs. Research advancements in the past 10–15 years have resulted in numerous mAB-based therapies that have been approved for inflammatory and neoplastic diseases. Infectious diseases have not been included in approved treatments. Although many mAb products targeting infectious diseases are in different stages of development, to date, one mAB-based product, Synagis (palivizumab), is currently approved for use in infectious diseases (RSV) [219], while two mAbs Abthrax (raxibacumab) and Anthim (obiltoxaximab) have been approved under the FDA's Animal Efficacy Rule for treatment of inhalation anthrax [8]. For treatment of Ebola infection, the single mAb mAB114 and Zmapp, a cocktail of three "humanized" mABs, have advanced in product development and are being tested for efficacy in the ongoing Ebola outbreak in the Democratic Republic of the Congo (NCT03719586, www.clinicaltrials.gov).

It is clear that mAbs offer a highly specific, potent, and generally safe platform for antimicrobials and may be a useful alternative to immune plasma. It is imperative to find appropriate niches in infectious diseases, specifically those caused by biothreat agents, where new antibody-based treatments could prove to be efficacious [220]. Table 7.5 summarizes key research in antibody therapy across different bacterial and viral families of some current biothreat agents. The utilization of mAB therapy for the prophylactic or therapeutic treatment of biothreat agents varies depending on the agent. In all cases however, the challenge for the development of effective therapeutic antibodies against viruses is the viruses' heterogeneity and mutability. A related problem is the low binding affinity of cross-reactive antibodies that are capable of neutralizing a variety of primary isolates. Finally, the cost of large-scale production of mABs is a limiting factor for continued use.

A solution to the challenges with viral mutagenicity may be found in the identification of potent new mAbs that target highly conserved viral structures, which are critical

for virus entry into cells. Alternatively, utilization of combination therapy, whereby, a cocktail of several mAbs may be used or mAbs may be combined with other drugs, such as antiviral compounds, may overcome mutagenicity issues. These areas of research will continue to be a major focus of biothreat agent therapeutic research [221].

4.3 Antiviral medical countermeasures

For countermeasures against lethal viral infections (i.e., category A), Table 7.3 lists reported studies in either mice or NHPs that have shown significant benefits to survival in challenge models. The table also includes in vitro potencies, viral strains, specific animal species, dosing regimens, routes of administration, PKs, and benefits to survival—data necessary for the reader to relate in vitro potency to in vivo efficacy, assess/interpret results, and make comparisons. The corresponding chemical structures are provided in Fig. 7.6. Table 7.4 displays the status/results of clinical trials for therapeutics used for the treatment of infections caused by EBOV and LASV. Noteworthy, most of these clinical trials were underpowered without appropriate controls and hence results may be speculative.

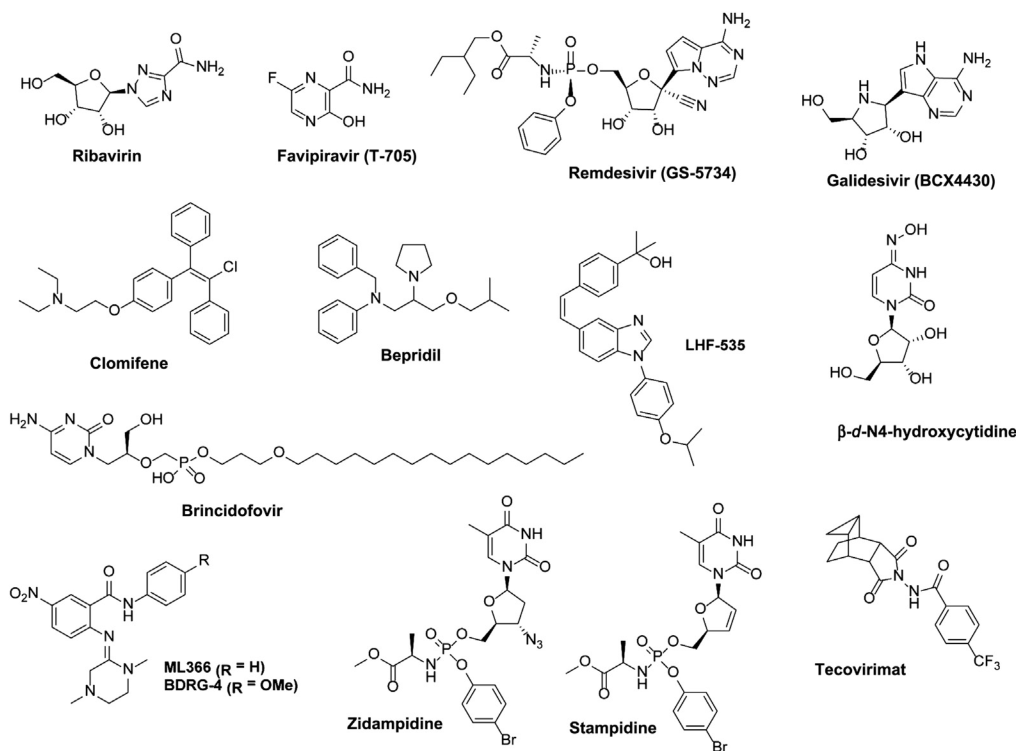

Figure 7.6 ***Structures of small molecule antiviral therapeutics.***

4.4 Combination therapies

Combination therapies are an excellent approach to improve treatment outcomes, shorten treatment duration, and overcome microbial resistance mechanisms caused by biothreat pathogens. Combination therapy may incorporate antibiotics or antivirals with HDT or antibody therapy at rationally designed treatment schedule. In this way the usage of multiple treatment modalities can synergize to optimize the mechanism of action of biothreat-targeted therapies. Table 7.6 includes combination therapies that have been used to treat each biothreat bacteria. In the case of viral infections, while combination therapy has been used for treatment of patients with human immunodeficiency virus (e.g., combination of nucleoside, nonnucleoside, protease, and/or host-targeted inhibitors) or chronic hepatitis C virus infection (e.g., combination of polymerase and RNA-binding protein NS5A inhibitors), to date there are no reported studies for biothreat viral agents. Several β lactam antibiotic drugs have been able to overcome deactivation when delivered in combination with inhibitors that target extended-spectrum β lactamases (enzymes that are overexpressed in the MDR pathogens, inactivate the β lactam antibiotic by cleaving the β lactam ring and thus one of the major contributors of antibiotic resistance). The β lactam/β lactamase inhibitor combination drugs that have been FDA-approved include Augmentin XR (amoxicillin/clavulanate combination), Unasyn (ampicillin/sublbactam combination), and Zosyn (piperacillin/tazobactam combination). In the case of biothreat bacteria, the combination of Ceftolozone and tazobactam exhibited increased in vitro susceptibility to a variety of clinical, environmental, and animal strains of *B. pseudomallei*, but to date it has not been evaluated in in vivo efficacy studies [222].

5 Unique preclinical challenges

Challenges to developing countermeasures against biothreat agents are many, but some of the unique and key challenges are PK differences in healthy versus infected subjects, mapping the biodistribution of the countermeasure to the biodistribution of the pathogen, and limited opportunities to run randomized, controlled clinical trials. Preclinical studies typically require a PK study in healthy animals to guide dose selection prior to testing a countermeasure in an animal model of infection. In that regard, it is critical to understand what cells and tissues the pathogen is infecting over time so that countermeasures can be properly designed to reach infected tissue. For example, countermeasures against pathogens causing encephalitis require drug to reach the central nervous system (CNS). In contrast, EBOV was found to infect lymph nodes, spleen, and liver in NHPs 2–3 days following viral challenge, and by days 5–6 the virus was detected throughout the body (Fig. 7.7) [217]. Thus if one was designing a countermeasure against EBOV infection, it would likely require a wide tissue distribution in order to be effective. To complicate things further, infected animals often have altered metabolizing enzymes

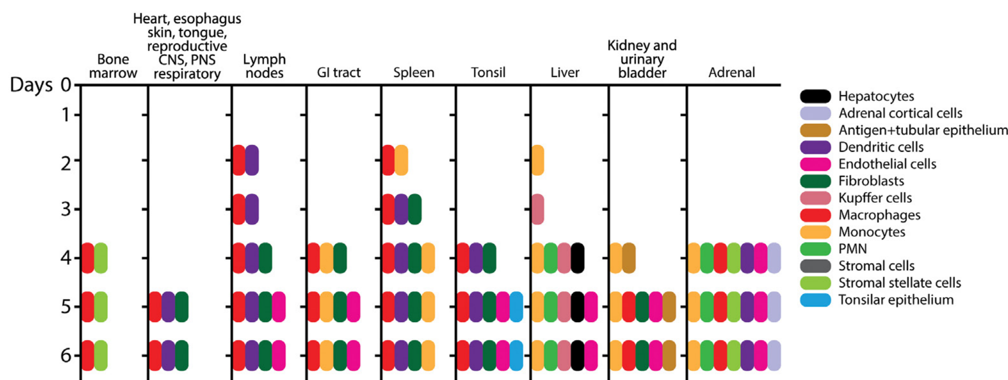

Figure 7.7 ***Time scale dissemination of EBOV and the infection of the varying cell types in the different tissues of NHP.*** Studies have described the dissemination of EBOV in tissues of infected NHP and specifically identified infected cell types during the time postinfection [217,224]. *EBOV*, Ebola virus; *NHP*, nonhuman primate.

(e.g., cytochrome p450s) [223], tissues, and barriers (e.g., blood–brain barrier) making drug exposure difficult to predict. Running PK experiments in the presence of infection would eliminate many of these variables, but this is seldom done for countermeasures to biothreat agents, since it requires running these experiments in biocontainment labs.

6 Clinical trials and the animal rule

Because biothreat pathogens cause infrequent human cases and outbreaks in generally remote areas of the world, planning a traditional human clinical trial with large numbers of participants is not feasible. Even when the West African Ebola outbreak of more than 28,600 cases was unfolding in 2014–2016, and now that there is a large outbreak unfolding in the Democratic Republic of the Congo, the amount of clinical efficacy data that have been collected for EBOV therapeutics is quite limited. The limitations are due to difficulty of performing clinical research in a remote outbreak setting where cultural, geographical, and political barriers may hinder or halt trial planning [225]. The bulk of the efficacy data for Ebola published in the literature has been garnered through animal studies. To enable product development for viral, bacterial pathogens as well as for chemical, toxin, and radiological threat agents for which outbreaks or cases are sparse, the FDA issued an Animal Rule, codified 21 CFR 314.600 in 2002, that proposes to permit consideration of product development and efficacy data obtained from animal studies for drug licensing, in lieu of human clinical trials when such trials would be unfeasible or unethical [226].

Since introducing the Animal Rule in 2002, the FDA has approved more than a dozen products, including several therapeutics for anthrax, plague, botulinum toxin, and smallpox [8,29]. The Animal Rule does not provide an expedited pathway to FDA

approval for drugs and can certainly be more challenging than traditional drug development pathways. The developer must compile a significant body of data to prove efficacy of the drug against the target therapeutic indication. First in 2009, and updated now into a formal document published in 2015 [226], the FDA has released guidance for Industry describing critical data elements required for animal efficacy studies for drug approval under the Animal Rule: (1) the pathophysiology of disease and the mechanism of action by which the drug prevents or ameliorates disease must be reasonably well understood; (2) it is desired that the efficacy must be demonstrated in two animal species, although multiple studies in one species can be acceptable if the animal model is sufficiently well characterized and accurately predicts the human response; (3) the animal study end point must be clearly related to the desired human efficacy end point, such as enhancement of survival; and (4) PK and pharmacodynamics data must be generated in the animal studies to allow selection of an effective dose in humans.

Under the Animal Rule, efficacy studies are expected to demonstrate that drug effectiveness in animals reliably indicates efficacy in humans. Thus while traditional human clinical efficacy studies require demonstration that the therapy is effective, the Animal Rule imposes an additional burden on investigators to establish a drug candidate's mode of action in at least one animal model that reproduces accurate human disease pathology. Further, the Animal Rule outlines considerations for the development of the model(s), to include the use of an isolate of the etiologic agent that was known to cause human disease (e.g., agent was isolated from a fatal human case if it is a lethal disease, such as Ebola) [227]. There is also a requirement that the infection model using the chosen pathogen strain must present the same or similar pathophysiology as the human disease. Definitive animal model efficacy evaluations should be performed only after careful model development studies have been performed and accepted by the regulators. These studies are known as natural history studies and are carefully designed to investigate and describe the course of the disease in the animal species, through clinical, serological, and histopathological evaluations, to compare the features of the disease in the model to the features of disease in human cases. It is important to consider the route of pathogen exposure (nasal, oral, and aerosol routes) to the animal because this will model the natural or unnatural modes of exposure predicted for humans, where a biorelease would constitute an unnatural exposure. A dose of challenge agent that is thought to be predictive of the human exposure level in a biorelease scenario should be used to develop the model, and that dose should be well characterized and reproducible by a quantitative measure. The route of drug delivery, dose administration timing, and treatment regimen in response to a biorelease scenario must also be considered when designing the animal model studies for a drug under development for such an indication. It is possible that a biorelease scenario would not be immediately known, and a period of time might pass before people begin to develop symptoms. Studies evaluating the cutoff time for drug to still be effective, and what are the triggers for treatment should be investigated in the animal model.

Animal Rule pivotal efficacy studies are essentially performed in place of traditional phase 3 clinical studies, so they must be done in the containment laboratory under a quality system [226]. Use of FDA Good Laboratory Practice or other comparable quality system with high levels of documentation and data integrity is paramount, so data packages can withstand regulatory review and audit [228]. The studies must be designed so that the program will collect the same results and conclusions one would expect from a well-designed traditional phase 3 trial, but in addition a pivotal animal efficacy study must describe a mechanism of action for the treatment modality to prevent or block disease or tissue infection and damage [227]. The pathologic mechanism needs to be consistent and well understood across both the human and animal models, such as the mechanism of pathogen entry into the target host cell, toxicological mechanism of lethal factors, or germination of spores and dissemination of bacterial infection in target cells and tissues, all of which may be mechanisms the drug under study is known to block; this must be proven in the animal model.

Products developed under the Animal Rule are subject to postmarketing or field studies when the product is actually used in the scenarios for which it was developed, and this is required to verify a product's clinical benefit [8]. Part of the approval process is a requirement to have postmarketing study plans in place, for quick execution should an event occur in which the drug would be field tested. Approval may also come with restrictions for off-label use, distribution, or access. Actual use will also come with requirements to inform patients of the conditions under which the drug was approved by virtue of only animal efficacy data, making them informed consumers as to the risks of possible nonefficacy or unknown effects in cases of human disease.

7 Summary and conclusion

With the advancement of systems and synthetic biology and the ease of genetic modification, biothreats are becoming more complex and there is a growing need for novel treatments that can have broad-spectrum activity against new, remerging, and engineered pathogens. Developing novel countermeasures that can effectively treat and prevent massive casualties is an ongoing challenge that remains a central priority for future research. The development of novel therapies relies on an improved understanding of the host–pathogen interactions. Key virulence factors have been identified and targeted for potential treatment options, including biofilm and T3SS inhibitors for bacterial infections, and viral entry or polymerase inhibitors for viral infections. Combining HTS with systems biology provides a robust, coordinated approach to identifying therapeutic targets. Since stand-alone antibiotics or antivirals may not be sufficient to overcome resistant or engineered biothreat infections, a focus on combination therapy, antibodies, and HDTs is the key countermeasure. Although many challenges are faced when developing novel therapies for biothreat pathogens and no FDA-approved HDTs are yet available

for treatment, there is potential for novel small molecule host-targeted immunomodulators to be developed. Screening of FDA-approved drugs is a powerful approach to possibly repurpose drugs for new disease indication and/or identify compounds that are safe and effective in humans, which can also have antibacterial or antiviral capabilities. Three FDA-approved drugs have shown potential in mice against pneumonic plague [207]. Serious challenges still remain with the prevalence of antibiotic resistance that jeopardizes the effectiveness of our current treatment options for bacterial threats. In addition, the complex intracellular life cycle of many biothreat pathogens requires therapeutics that can penetrate the host cell. There are limited FDA-approved viral countermeasures for prevention and treatment. None of the filoviruses or henipaviruses has approved therapeutics or vaccines available for human disease. Some vaccines exist for new world alphaviruses, but no current therapeutics are effective for treatment after infection. Focused efforts using HTS to develop novel, effective, and broad-spectrum medical countermeasures will provide a robust response capability against rapidly evolving biothreats.

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